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Baculovirus display for discovery of low-affinity extracellular receptor–ligand interactions using protein microarrays

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ABSTRACT

When used in conjunction with multivalent protein probes, protein microarrays offer a robust technology for discovery of low-affinity extracellular protein–protein interactions. Probes for receptor-matching screens generally consist of purified extracellular domains fused to affinity tags. Given that approximately two-thirds of extracellular proteins are transmembrane domain-containing proteins, it would be desirable to develop a system to express and display probe receptors in a native-like membrane environment. Toward this end, we evaluated baculovirus display as a platform for generating multivalent probes for protein microarray screens. Virion particles were generated displaying single-transmembrane domain receptors BTLA, CD200, and EFNB2, representing a range of affinities for their interacting partners. Virions directly labeled with Cy5 fluorophore were screened against a microarray containing more than 600 extracellular proteins, and the results were compared with data derived from soluble Fc protein or probe-coated protein A microbeads. An optimized protocol employing a blocking step with a nonrelated probe-expressing control baculovirus allowed identification of the expected interactions with a signal-to-noise ratio similar to or higher than those obtained with the other formats. Our results demonstrate that baculovirus display is suitable for detection of high- and low-affinity extracellular protein–protein interactions on protein microarrays. This platform eliminates the need for protein purification and provides a native-like lipid environment for membrane-associated receptors.

Extracellular protein–protein interactions (ePPIs)[1] are often transient and low affinity, making them difficult to detect in screening assays [1–3]. To enhance detection, a number of probe formats that impart significant avidity through multimerization have emerged. Multimeric fusion tags such as the Fc portion of human IgG (dimer) and the coiled–coil sequence from the rat cartilage oligomeric matrix protein (pentamer) have been employed [4–7]. Furthermore, streptavidin- or protein A-coated microbeads have been applied to detect weak interactions [8]. Recently, it was shown that protein microarray technology, when combined with these multivalent probes, is effective for studying low-affinity ePPIs [9–12].

In this study, we have investigated the application of recombinant baculovirus (BV) display as an alternative probe format for protein microarrays that allows for expression of full-length transmembrane domain-containing proteins in a native-like lipid bilayer and eliminates the need to directly purify the recombinant protein using an affinity tag. BV virions are enveloped in an external phospholipid bilayer derived from the host cell surface during egress from infected cells. By this process, BV virions selectively incorporate the major BV glycoprotein gp64 and, presumably through passive acquisition, other overexpressed cell surface proteins [13–17]. BV display has successfully been used in antibody development, particularly with hard-to-produce multi-transmembrane domain antigens [18–21]. Sakihama and coworkers successfully used BV display to detect several coreceptor interactions, including a low-affinity example, in enzyme-linked immunosorbent assay (ELISA) and cell-binding formats [22]. These results suggest that the BV virion itself can serve as a multivalent probe capable of binding to low-affinity binding partners and, therefore, might be well suited for extracellular receptor–ligand discovery using protein microarrays.

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[1] *Abbreviations used:* ePPI, extracellular protein–protein interaction; BV, baculovirus; BTLA, B and T lymphocyte attenuator; Eph, erythropoietin-producing hepatocellular; EFNB2, ephrin-B2; RIC, restriction-independent cloning; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBST, PBS with 0.05% Tween 20.

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To test this idea, we selected three well-characterized coreceptor interactions: CD200/CD200R, BTLA/TNFRSF14, and EFNB2/Eph. These cell surface receptors possess a range of binding affinities to their ligands. For example, B and T lymphocyte attenuator (BTLA) and TNFRSF14 interact with high affinity ($K_D \sim 6–25$ nM) [23]. In contrast, the immune modulators CD200 and CD200R1 interact with much lower affinity ($K_D \sim 1$ μM), and efficient detection of the interaction on microarrays requires multivalent probes [5,8,9]. The ephrin (Eph receptor interacting proteins) ligands and Eph (erythropoietin-producing hepatocellular) family of receptor tyrosine kinases are divided into A and B classes based on binding preferences [24]. Within the same class, ephrins interact with Eph receptors promiscuously and with high affinity. For example, the intra-class interaction of ephrin-B2 (EFNB2) with EphB4 has a higher affinity ($K_D = 40$ nM) compared with the inter-class interaction of EFNB2 with EphA4 ($K_D = 0.2–10.8$ μM) [25–27]. Using these test cases, we show here that BV display is a suitable approach for screening protein microarrays with phospholipid membrane-associated protein probes.

Materials and methods

Recombinant BV construction

Recombinant BV constructs were generated by restriction-independent cloning (RIC). For each construct, separate polymerase chain reactions (PCRs) were prepared with two primer sets that were appended with the following bases to incorporate sticky ends during PCR amplification: (i) forward A (BamH1) 5′-p-GATCC + reverse A (EcoR1) 5′-CCC and (ii) forward B (BamH1) 5′-C and reverse B (EcoR1) 5′-p-AATTCCC. The PCR products were cleaned, denatured, reannealed, and Dpn1 treated to generate PCR inserts with sticky ends for ligation into BamHI and EcoR1 linearized RIC vector with a pAcGP67A backbone (Pharmingen) containing a C-terminal Flag epitope tag. All BV constructs were confirmed by DNA sequencing. The amino acid boundaries for receptor–C-terminal Flag fusions were BTLA (K2-S289), CD200 (E2-R267), and EFNB2 (A2-V333).

Preparation of budded BV particles

Sf9 cells (400 ml at 2×10^6 cells/ml) were infected with recombinant BV sufficient to produce a high titer viral stock (ranging from 5.5e8 to 1.5e9 pfu/ml). Next, 72 h post-infection, cells and large debris were removed and BV particles were isolated from the supernatant by spinning at 38,000*g* (16,000 rpm) for 1.5 h in a Beckman Centrifuge (JLA-16.250). The resulting BV pellets were suspended in 4 ml of phosphate-buffered saline (PBS) containing protease inhibitor cocktail (complete, ethylenediaminetetraacetic acid [EDTA] free, Roche), creating a 100× concentrated BV suspension. Because BV instability has been associated with storage at 4 °C as a result of viral particle aggregation (especially with high viral titers) [28], and because of our own observations of decreased activity after 1 month, we recommend that BV stocks be used soon after their production to ensure no loss of protein function.

The expression of recombinant proteins in 100× BV solution was confirmed by fluorescent detection on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels using a Typhoon imager (GE Heathcare). Either anti-FlagM2-FITC (Sigma) or anti-gp64-PE (Expression Systems) was used. BV samples were incubated with 1% FC12 (*n*-dodecylphosphocholine) for 1 h at 4 °C and then spun at 16,000*g* (14,000 rpm) for 35 min. A portion of the soluble fraction was mixed with 2× SDS–PAGE sample loading buffer (without reducing agent), containing 20 mM urea, without boiling. After resolving by SDS–PAGE, final detection was made using a Typhoon imager.

Protein microarray

Protein microarrays were prepared as described previously [9,11]. Briefly, recombinantly produced and purified protein samples were immobilized in duplicate spots on epoxy-derivatized slides (Nexterion Slide E, Schott). Between each protein sample on the microarray, BSA–Cy3 was printed in order to visualize the spot locations and to control for sample carryover. Slides were blocked using a fogging device with ZeptoMARK blocking buffer BB1 (Zeptosens), followed by immersion in 5% skim milk (LP0031, Thermo Fisher) overnight. Slides were then washed in PBST (PBS with 0.05% Tween 20) and stored at −20 °C in 40% glycerol/PBS (v/v). Immediately before use, slides were warmed to room temperature and briefly rinsed with PBST.

The BV particles displaying BTLA, CD200, or EFB2 were labeled with Amersham Cy5 monoreactive dye (GE Healthcare) in PBS at a ratio of 5 μl of Cy5 dye to 100 μl of the 100× BV suspension at room temperature for 15 min. The reaction was diluted by the addition of 1 ml PBS and the BV was pelleted at 21,000*g* for 15 min at 4 °C. The BV pellet was resuspended in 500 μl of PBS and pelleted again, followed by resuspension in a final volume of 100 μl PBS. Immediately prior to probing the protein microarray, the Cy5-labeled BV was diluted 10-fold in PBS/5% skim milk, PBS/5% skim milk supplemented with 0.35 M NaCl, or PBS/5% skim milk supplemented with unlabeled control BV diluted 2.5-fold. Protein microarray slides were processed using an automated a-Hyb hybridization station (Miltenyi Biotech) at 15 °C using the

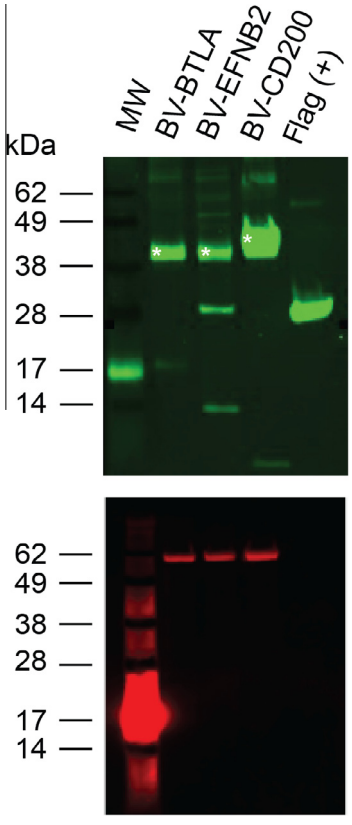

Fig.1. Visualization of membrane proteins expressed on BV virions. Solubilized BV virions were separated by SDS–PAGE, and the gels were probed with anti-Flag-FITC (top) or anti-gp64-PE (bottom) and visualized using a Typhoon fluorescence imager. Roughly equivalent amounts of BV virion (10 μl of viral stock) were loaded, as indicated by the gp64 fluorescence intensity. A Flag-tagged protein control (10 ng) was included (right-most lane), and molecular weight (MW) markers were run on the left-most lane (a molecular weight marker running at 17 kDa showed background fluorescence in this assay). Full-length C-terminal Flag-tagged BTLA, EFNB2, and CD200 are indicated by white asterisks.

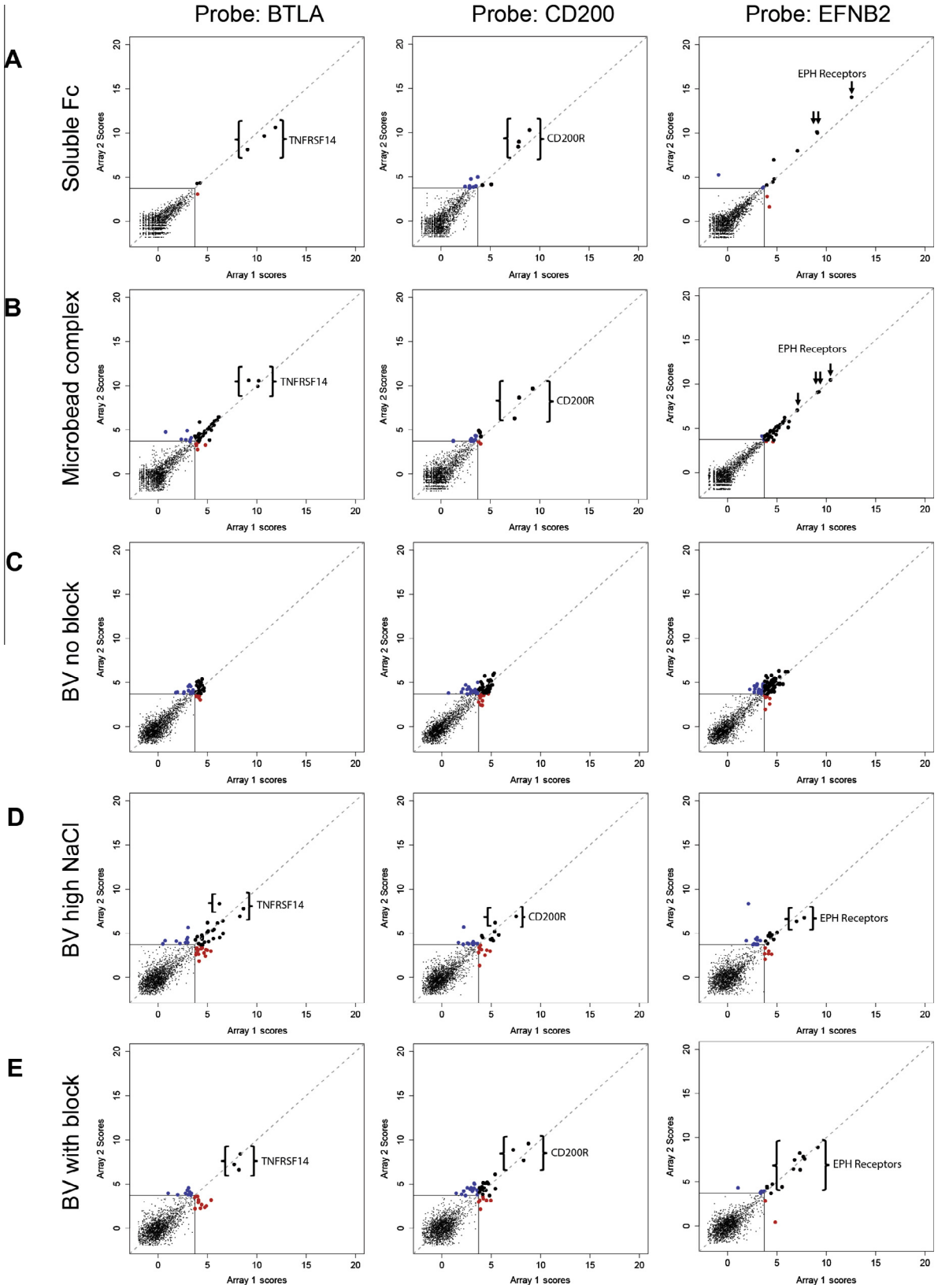

Fig.2. Protein microarray screens using Fc proteins, microbead complexes, or BV virions. Intersection plots representing data from two replicate microarrays are shown for BTLA, CD200, and EFNB2 probes. The plots represent data obtained using the following formats: (A) soluble Fc; (B) microbead complex; (C) BV probe without BV control blocking; (D) BV probe with increased NaCl (500 mM) and without BV control blocking; (E) BV probe with BV control blocking. The hit cutoff is indicated by the square on the lower left quadrant of the plot and represents the 0.0001 probability cutoff from a fitted normal distribution. Hits beyond the cutoff are shown as larger dots, with the blue and red representing non-intersection hits (exceeded the cutoff on one slide only) and the black dots representing intersection hits (exceeded the cutoff on both slides). Data are representative of two independent BV probe lots tested for each receptor.

following staining protocol: wash five times with PBS for 1 min (step 1), load 200 µl of unlabeled control BV diluted 10-fold in PBS/5% skim milk and incubate for 30 min (step 2), wash five times with PBS for 1 min (step 3), load 200 µl of Cy5-labeled BV in PBS/5% skim milk in the presence of unlabeled control BV and incubate for 30 min (step 4), and wash five times with PBS for 1 min (step 5). Steps 2 and 3 were omitted and step 4 was adjusted for binding assays run in the absence of unlabeled control BV blocking. Buffers were changed for all steps for high-salt studies. For comparison with BV display, recombinant Fc fusion proteins for BTLA, CD200, and EFB2 were also probed against the extracellular protein microarray by direct Cy5 labeling or through the formation of protein A microbead–Fc fusion complexes with Cy5–IgG as a carrier for fluorescent dye as described previously [11]. The slides were rinsed in PBS, spun dry by centrifugation, scanned with a GenePix 4000B scanner (Molecular Devices) for Cy3 and Cy5 fluorescence by exciting at 532 and 635 nm, and then analyzed using GenePix Pro 6.0 software (Molecular Devices). Signal-to-noise ratios were defined as the average background-subtracted fluorescent signal (F635–B635) for duplicate spots divided by the average spot intensity (F635–B635) over the entire slide. Scores were calculated according to Ramani and coworkers [9].

Results and discussion

To explore the possibility of using recombinant BV virions as probes for screening ePPIs on protein microarrays, Sf9 insect cells were infected with recombinant BV, individually encoding BTLA, CD200, or EFNB2. An intracellular C-terminal Flag tag was incorporated into each construct to allow detection by immunoblot while leaving the extracellular domain untagged. The BV fractions were recovered and analyzed for expression using an anti-Flag antibody and anti-gp64 as a loading control (Fig. 1). A predominant band of approximately 40 kDa was observed for BV-BTLA (lane 2), which is consistent with the expected size of 31 kDa plus glycosylation. BV-EFNB2 (lane 3) displayed one higher molecular weight band and two smaller molecular weight bands. This pattern was reproducible in independent preparations. The most prominent component, running at approximately 40 kDa, likely represents the full-length receptor (35 kDa expected), and the approximately 30- and 14-kDa bands are likely N-terminal proteolytic truncations. BV-CD200, with a theoretical molecular weight of 32 kDa, migrated at approximately 45 kDa (lane 4). The larger size of CD200 displayed on BV is in agreement with previously published immunoblots of CD200 in transfected HEK293 cells and is likely due to extensive glycosylation [29].

We previously developed an extracellular protein microarray containing 1334 protein samples representing 686 genes [9]. Using these microarrays, we screened either Cy5-labeled soluble Fc protein or unlabeled Fc protein in complex with protein A microbeads (with IgG–Cy5 as a carrier of the fluorescent dye). The probe–Fc fusion proteins were screened on duplicate microarrays, and the top hits were identified (Fig. 2A and B; see also Table S1 in online supplementary material). As expected, BTLA and CD200 bound to the three independently expressed and purified lots of TNFRSF14 and CD200R present on the microarrays, respectively. The screen using soluble EFNB2-Fc displayed strong binding signals to three (EphA7, EphB4, and EphA4) of seven highly immobilized Eph receptors present. One additional interaction, corresponding to a protein lot of EphB6, was identified when the microbead complex was used. We should note that the relative fluorescent signals (and derived hit scores) for different protein lots are not necessarily reflective of interaction affinity given that many factors, such as protein activity and immobilization levels on the microarray, can play a role in determining the observed signal [9]. The scores for individual protein lots, however, should be comparable across assays.

Next, recombinant baculoviruses (BV-BTLA, BV-CD200, and BV-EFNB2) were used to screen the protein microarrays. Each virus was directly labeled with Cy5 and, as with the Fc fusions and bead complexes, was screened in PBS containing 5% skim milk to block nonspecific interactions (Tween 20 detergent was omitted to prevent possible disruption of the BV particles). In contrast to the control screens described above, the expected interactions observed using the BV particles were not clearly distinguished from noise (Fig. 2C and Table S1). For example, the scores for the BV-BTLA hits (3 lots of TNFSFR14) averaged 4.8, whereas the average for the next 10 highest scores was 4.4. The scanned microarray image for the top 3 hits from the EFNB2 screens (Fig. 3) clearly shows an increase in nonspecific binding.

To investigate whether this background was due to nonspecific ionic interactions, we increased the salt concentration in the assay buffer to 500 mM NaCl and rescreened the samples (Fig. 2D and Table S1). For each case, the increased salt did result in reduced nonspecific binding. However, the distinction between the true hits and the noise was lower in each case compared with the screens with microbead complexes. For example, the average score for the BV-BTLA/TNFSFR14 hits was 7.7 versus an average of 5.6 for the next top 10 highest scores. In the BTLA–Fc microbead complex screen, the TNFRSF14 hits averaged 10.1, whereas the next top 10 highest scores averaged 5.8.

Because increasing salt did not adequately rescue signal-to-noise ratios, we investigated an alternative blocking strategy. In this case, an unlabeled nonrelated BV particle was used as a nonspecific binding blocking agent. The blocked BV-BTLA screen produced strong signals with clear separation from the noise without needing to increase the salt concentration (Figs. 2E and 3; see also Fig. S1 and Table S1 in supplementary material). The TNFRSF14 hits for BV-BTLA averaged 7.8, whereas the next top 10 scores averaged 3.5. Similarly, BV-EFNB2 showed strong binding signals to the same 4 ephrin receptor lots identified as hits using the microbead complex, and 3 additional ephrin lots were clearly detected in the top hits.

In summary, we have investigated the use of BV display for application in extracellular protein–protein interaction screening using protein microarray technology. Specific binding was successfully demonstrated between single-transmembrane domain receptors displayed on BV virions and their ligand(s) present on the

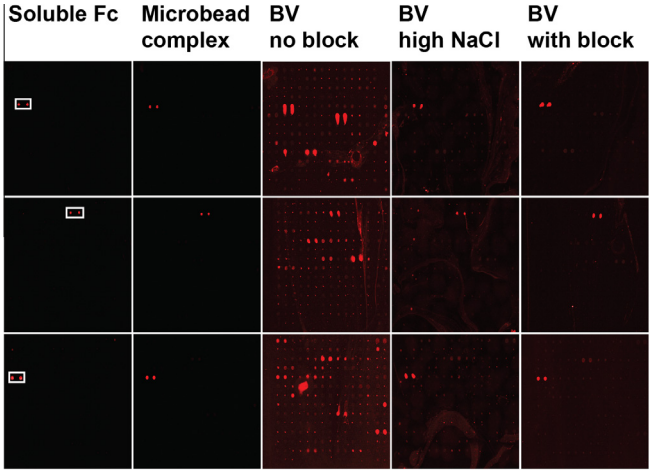

Fig.3. EFNB2 screen images. Images are shown for individual sub-grids (F3, G1, and L1 in Fig. S1 of the supplementary material) containing the top 3 hits in the EFNB2 probe screens. Cy5 (red) signal represents EFNB2 binding. Duplicate spots for the 3 expected interactions are outlined in white boxes in the left-most panel.

protein microarray. It is likely that the avidity provided by multiple copies of receptors on each BV virion contributed to the ability to detect low-affinity interactions. A potential advantage of BV display is that the receptors may retain lateral mobility throughout the lipid bilayer, allowing for receptor clustering that can be important for certain types of receptor interactions [30]. The BV display approach also eliminates the need to directly purify the membrane associate protein and allows for an untagged, native-like environment. A simple centrifugation step is sufficient to separate and recover the budded virions from the infected insect cells, and only a small amount of BV is required for screening. Consequently, this approach can be easily miniaturized and scaled, allowing multiple BV probes to be generated in parallel. BV display may also be useful for screening intact heteromeric receptors or for probing low-affinity carbohydrate interactions on glycan arrays [31]. Importantly, BV particles are known to incorporate multi-transmembrane domain proteins [20]; therefore, BV display may enable their screening on protein microarrays.

Competing interests

The authors are all employees of Genentech/Roche.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.03.015.

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